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Excreted Metabolites of Gonadal Steroid Hormones and Corticosterone in Greylag Geese (*Anser anser*) from Hatching to Fledging

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Steroid hormones play major roles in the organization of the phenotype and in the activation of behavior. From hatching to fledging, they are involved in growth, development, and learning. We investigated the relationship between the ontogenetic patterns of steroid hormones and the sexual and social development of greylag goslings (*Anser anser*). Two groups of individually marked goslings ($n = 10/5$) were hand-raised under near-field conditions. 17β-OH-androgen (AM), estrogen (EM), and corticosterone (BM) immunoreactive metabolites were measured noninvasively by enzyme immunoassay of individual fecal samples. Feces were regularly sampled from hatching to fledging. All excreted steroids were found to peak at hatching and to decrease thereafter. Gonadal steroids fluctuated more than BM, which remained at low levels throughout ontogeny after a slow decrease during the first 20 days. The pattern of BM is discussed in relation to learning processes (i.e., filial imprinting) and social stress. It is suggested that high initial BM may constrain energy allocation to growth. AM increased around the age of 20 days, when the feathers start growing, and later, together with EM, at the age of 40 days. These elevated values of gonadal steroids are discussed in relation to the sensitive phase of sexual imprinting. Females show higher EM levels than males throughout ontogeny. Furthermore, the ratio of excreted estrogen to androgen (EM/AM) of females before fledging correlates with the number of hatched and fledged goslings in their first years of reproduction. In conclusion, our data suggest a role for steroid hormones in the modulation of behavioral and morphological development in the precocial greylag geese, in agreement with the organizational–activational hypothesis.

Key Words: Anser anser; feces; noninvasive endocrine monitoring; corticosterone; gonadal steroids; ontogeny; development; growth.

INTRODUCTION

During early stages of life, steroid hormones affect the phenotypic development of brain and secondary sexual characteristics (McLusky and Naftolin, 1981). During puberty and adulthood they activate behaviors which had been organized earlier during ontogeny (organizational–activational hypothesis; Phoenix *et al.,* 1959; Adkins-Regan, 1983; Arnold and Breedlove, 1985).

Prehatch exposure to (maternal) steroids may affect individual behavioral phenotypes (Schwabl, 1993, 1999). The fetus also synthesizes steroids from abundant precursors in the yolk (cholesterol, progesterone; see McNabb *et al.*, 1998). In parallel to mammals at birth (Baum *et al.*, 1988 in rats, *Rattus* sp.; Hodges and Hearne, 1978 in nonhuman primates; Forest and Cathiard, 1975 in humans, *Homo sapiens;* see also Nelson,

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1995), high levels of steroid hormones at hatching have been described in altricial and precocial birds (Proeve, 1983 and Adkins-Regan *et al.*, 1990 in zebra finches, *Taeniopygia guttata castanotis;* Williams *et al.*, 1987 in starlings, *Sturnus vulgaris;* Ottinger and Brinkley, 1979 in Japanese quails, *Coturnix coturnix japonica;* Tanabe *et al.*, 1983 in mallards, *Anas plathyrhynchos;* Tanabe *et al.*, 1986 in domestic chickens, *Gallus gallus*). The functional significance of these high perinatal steroids remains unclear. However, a relationship with the drastic environmental changes occurring at birth or at hatching and with essential learning processes may be proposed.

After hatching, sexual imprinting (Immelmann, 1972) and sexual differentiation of copulatory behavior (Adkins-Regan, 1987) occur during sensitive periods. In passerine birds, males learn their song from adult models (Kroodsma, 1978). All these processes depend on gonadal steroids (Adkins-Regan *et al.*, 1990). Despite marked differences between precocial and altricial species (Ottinger and Abdelnabi, 1997), manipulation of embryonic and early posthatch steroid hormones affects endocrine and behavioral responses in adult birds (e.g., several studies on Japanese quail, *C. coturnix japonica*, Adkins-Regan *et al.*, 1995; for a review see Balthazart *et al.*, 1996).

Systemic steroid hormone concentrations during early stages of life have been repeatedly measured (e.g., Balthazart *et al.*, 1983; Proeve, 1983; Tanabe *et al.*, 1983, 1986; Williams *et al.*, 1987; Schumacher *et al.*, 1988; Adkins-Regan *et al.*, 1990). However, repeated and regular blood sampling in hatchlings may be technically difficult and may feed back on the parameters being measured. Noninvasive sampling is an alternative that allows short sampling intervals. During the last few years, fecal steroid metabolites (androgen, estrogen, progesterone, and corticosterone) have been measured in greylag geese (*Anser anser;* Krawany, 1996) in the context of seasonal and social patterns (Kotrschal *et al.*, 1998; Hirschenhauser *et al.*, 1999a,b, 2000a). The method has been properly validated (Hirschenhauser *et al.*, 2000b; Kotrschal *et al.*, 2000).

In the present study, excreted steroid metabolites were measured noninvasively from hatching to fledging in a precocial bird, the greylag goose. Patterns of 17β-OH-androgen, estrogen, and corticosterone immunoreactive metabolites are suggested as a basis for the timing of behavioral and morphological development. In particular, we discuss their roles as markers for sensitive periods of learning, such as social and sexual imprinting. Furthermore, the potential value of estrogen / androgen ratio in females as a predictor for their forthcoming reproductive success is also discussed.

METHODS

Animals

For most of the year greylag geese are social birds (Lorenz *et al.*, 1978; Rutschke, 1982). Migrating and wintering flocks include family groups, pairs without offspring, and singletons (Elder and Elder, 1949). A nonmigratory flock of greylag geese was introduced into the Upper-Austrian valley of the river Alm by Konrad Lorenz in 1973 (Lorenz, 1988). The geese are unrestrained and subject to natural selection (Kotrschal *et al.*, 1992). The flock consisted of 120 individuals at the time of data collection. Individuals are marked with colored leg rings and are habituated to the presence of humans. Detailed life history information is available for all of them.

For scientific reasons, hand-raising is carried out every year in the same area where the goose families also raise their young. From hatching to fledging, the human foster parents stay permanently with their goslings and their daily spatiotemporal activity patterns follow those of the wild families. Hand-raised individuals come into contact with the flock shortly after hatching and are fully integrated by the time of fledging, establishing social bonds with goose partners and raising offspring, behaviorally indistinguishable from the goose-raised geese (own unpublished observations).

The present study is based on 15 hand-raised geese, from two different sibling groups ($n_1 = 10$; $n_2 = 5$), i.e., raised in the same way by two different foster parents. All individuals hatched between the end of April and the beginning of May 1993 (Table 1). The geese were individually marked with colored leg ribbons from hatching onward. This allowed sampling of droppings from known individuals.

TABLE 1
Focal Individuals and Their Life History Characteristics (Sibling Group, Date of Hatch, Sex When Known)

Sibling group	Individual	Date of hatch (dd.mm.yy)	Sex
1	1	04.05.93	f
1	2	04.05.93	m
1	3	06.05.93	m
1	4	06.05.93	f
1	5	06.05.93	m
1	6	10.05.93	f
1	7	02.05.93	
1	8	04.05.93	
1	9	06.05.93	
1	10	05.05.93	
2	11	25.04.93	m
2	12	25.04.93	f
2	13	25.04.93	f
2	14	25.04.93	
2	15	26.04.93	

Note. Individuals of unknown sex disappeared before the age of 3 years; m, male, f, female.

Data Collection

Gut passage time in geese is 2–3 h (Mattocks, 1971; Kotrschal *et al.*, 2000) and individuals may defecate more than once per hour. Steroid metabolites, produced in the liver, are excreted via hepatic and renal pathways (McDonald *et al.*, 1983; Peter *et al.*, 1996). Since separation of feces from uric acid (a small proportion of the sample in herbivorous birds) is not feasible in the goose droppings, they were analyzed together (Bercovitz *et al.*, 1978). As in other field studies, blood was not sampled and therefore, steroid plasma levels are unknown. However, in domestic geese injected with ^{3}H-labeled steroids, metabolite excretion peaked 1–2 h after injection (Krawany, 1996). Furthermore, in agreement with studies by Bishop and Hall (1991), Cockrem and Rounce (1994), and Palme *et al.* (1996), experiments with domestic geese showed deterministic relationships between plasma steroids and metabolites in the feces (Hirschenhauser *et al.*, 2000b; Kotrschal *et al.*, 2000). In addition, implantation consistently increased excreted metabolite concentrations (Frigerio *et al.*, 1997). Therefore we are confident that the feces contained an integrated, proportional record of the plasma steroid levels up to approximately 2 h before defecation.

To avoid the effect of diurnal variation (Schuetz *et al.*, 1997), individual fecal samples were collected during morning hours from hatching to fledging. Droppings were sampled daily during the first 2 weeks of life and then every second day until fledging (approximately at the age of 80 days). The samples were frozen at −20° within 2 h after collection. It was not always possible to sample all individuals on the same day. On average 47.53 ± 1.03 ($\bar{X}$ ± SE) samples per individual were collected over the entire period (total $N = 713$).

Fecal Steroid Assay and Data Analyses

Fecal samples (0.5 g) were extracted in methanol and hydrolyzed as described by Hirschenhauser *et al.* (1999a). Steroid metabolites were determined by enzyme immunoassay (EIA; Moestl *et al.*, 1987). Details about the procedure and cross-reactivities are published elsewhere (androgen: Hirschenhauser *et al.*, 1999b; estrogen: Hirschenhauser *et al.*, 1999a; corticosterone: Kotrschal *et al.*, 1998). The sensitivity of the assay was less than 0.3 pg/well, and concentration limits for reliable measurement ranged from 0.99 to 39.1 pg/well for androgen, from 3.25 to 47.9 pg/well for estrogen, and from 4 to 50 pg/well for corticosterone. Samples were assayed only once. Intra- and interassay variations were determined with homogenized pool samples. Mean intraassay coefficient of variation was 23.7% for androgen, 17.4% for estrogen, and 13.4% for corticosterone; mean interassay coefficient of variation was 18.6% for androgen, 19.8% for estrogen, and 14.1% for corticosterone. These relatively high values are characteristic for EIA procedures on feces; they are caused by the number of steps involved in the procedure, which add up to the total variation.

The abbreviations used follow the steroid nomenclature proposed by Kime (1995).

Patterns over the entire period were obtained and are discussed for intervals of 5 consecutive days starting at day 1 posthatching. The ontogenetic period considered (i.e., from hatching to fledging) was also investigated for the first 20 days, i.e., before the main feathers start growing (Hudec and Rooth, 1995), and the last 60 days before fledging. Since greylag geese are monomorphic birds and we did not use genetic sexing, the sex of the focal individuals was certain

TABLE 2
Effects of Age and Sex on Excreted Metabolites

Effect	AM	EM	EM/AM	BM
Age (5-day pattern) (two-way ANOVA)	**$F_{17,415}$ = 5.197** **P = 0.000**	**$F_{17,416}$ = 3.613** **P = 0.000**	**$F_{17,407}$ = 3.035** **P = 0.000**	**$F_{17,421}$ = 11.514** **P = 0.000**
Sex (5-day pattern) (two-way ANOVA)	$F_{1,415}$ = 2.456 P = 0.118	**$F_{1,416}$ = 15.218** **P = 0.000**	**$F_{1,407}$ = 17.533** **P = 0.000**	$F_{1,421}$ = 0.747 P = 0.388
Age*Sex (5-day pattern) (two-way ANOVA)	$F_{17,415}$ = 1.348 P = 0.160	$F_{17,416}$ = 1.343 P = 0.162	$F_{17,407}$ = 1.178 P = 0.279	$F_{17,421}$ = 0.420 P = 0.981
Age (20/60 days) (one-way ANOVA)	**$F_{1,415}$ = 20.591** **P = 0.000**	$F_{1,416}$ = 0.906 P = 0.342	**$F_{1,407}$ = 12.245** **P = 0.001**	**$F_{1,421}$ = 110.448** **P = 0.000**

Note. A two-way ANOVA was used to investigate the effect of age and sex on the pattern of steroid immunoreactive metabolites from hatching to fledging. Data concerning the first 20 days after hatching and the last 60 days before fledging were analyzed by one-way ANOVA. Significant results are in boldface.

only at the age of 3 years, when only 9 of the 15 geese were still alive. Therefore, when evaluating sex differences, we considered only four males and five females (Table 1).

Data were not normally distributed (Kolmogorov–Smirnov: androgen, $Z = 9.09$, $n = 694$, $P = 0.000$; estrogen, $Z = 6.571$, $n = 692$, $P = 0.000$; corticosterone, $Z = 5.202$, $n = 708$, $P = 0.000$); therefore a logarithmic (ln) transformation was performed. Individual arithmetic means were calculated for further analyses. To consider the simultaneous effect of different sex steroids (Ottinger and Abdelnabi, 1997), the ratio estrogen/androgen was calculated. Data were analyzed by the SPSS statistical program (Norusis, 1990). Results of all tests are given as two-tailed; Bonferroni post hoc corrections were performed whenever necessary (Rice, 1989). Kendall's τ was preferred for the correlation between the ratio estrogen/androgen and the number of hatched and fledged goslings in the females because it is suitable for the small n and it is quite robust with respect to extreme values (Zoefel, 1992).

RESULTS

Androgen

ANOVA demonstrated a significant effect of age but not of sex on the pattern of excreted 17β-OH-androgen immunoreactive metabolites (AM) from hatching to fledging (Table 2; Figs. 1a and 2a). Furthermore, AM was significantly higher during the first 20 days of life than during the last 60 days before fledging (Table 2; Fig. 3a).

Estrogen

ANOVA demonstrated a significant effect of both age and sex on the pattern of excreted estrogen immunoreactive metabolites (EM) from hatching to fledging (Table 2; Figs. 1a and 2b). The levels of EM during the first 20 days were not significantly different from those during the last 60 days (Table 2; Fig. 3b).

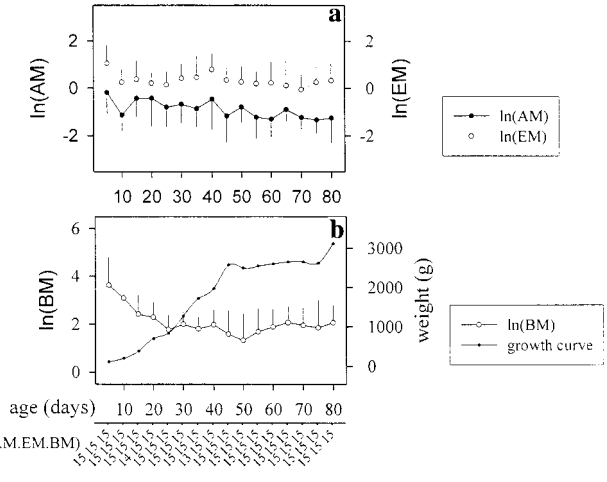

FIG. 1. Patterns of steroid hormones from hatching to fledging in 15 greylag goslings. Original values (ng steroid/g feces) have been logarithmic (ln) transformed and arithmetic means per 5 days are plotted. (a) AM (−SD) and EM (+SD); (b) BM (+SD). The growth curve in (b) as calculated for greylag goslings overlaps the pattern of BM.

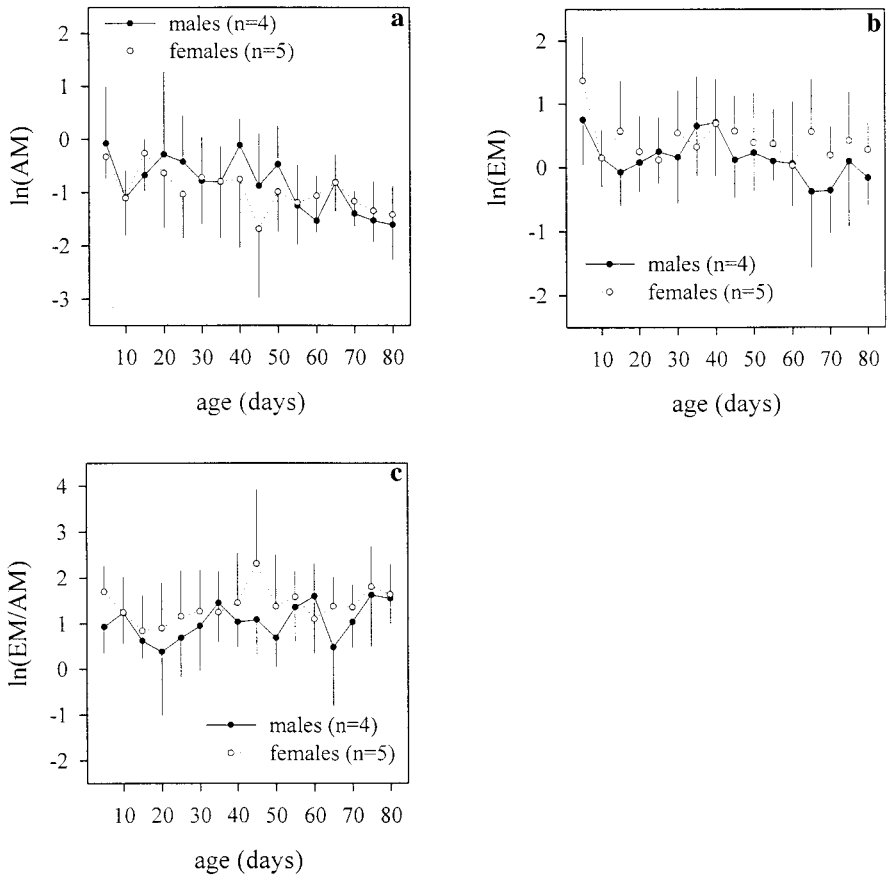

FIG. 2. Patterns of gonadal steroid hormones from hatching to fledging in four male and five female greylag goslings. Original values (ng steroid/g feces) have been logarithmic (ln) transformed and arithmetic means per 5 days are plotted. (a) AM in males (+SD) and females (−SD); (b) EM in males (−SD) and females (+SD); (c) EM/AM in males (−SD) and females (+SD).

Estrogen/Androgen

ANOVA demonstrated a significant effect of both age and sex on the pattern of the ratio of excreted immunoreactive metabolites of estrogen and androgen (EM/AM) from hatching to fledging (Table 2; Fig. 2c). Furthermore, EM/AM was significantly lower during the first 20 days than during the last 60 days (Table 2; Fig. 3c). Since early gonadal steroid levels may influence adult behavioral and reproductive patterns, the relationship between EM/AM and the reproductive output (i.e., total number of hatched goslings, total number of fledged goslings) in their first 6 years of life (1993–1999) was evaluated for the 5 females. EM/AM during the last 60 days before fledging and the number of hatched goslings were correlated (Kendall's $\tau = 0.949$, $n = 5$, $P = 0.023$; Fig. 4), as were EM/AM and the number of fledged goslings (Kendall's $\tau = 0.894$, $n = 5$, $P = 0.037$; Fig. 4).

Corticosterone

ANOVA demonstrated a significant effect of age, but not of sex, on the pattern of excreted corticosterone immunoreactive metabolites (BM) from hatching to fledging (Table 2; Fig. 1b). Furthermore, BM was significantly higher during the first 20 days of life than during the last 60 days before fledging (Table 2; Fig. 3d). As the mobilization of energy by the initial high BM may constrain the allocation of energy toward growth, the relationship between corticosterone and growth will be discussed (Fig. 1b).

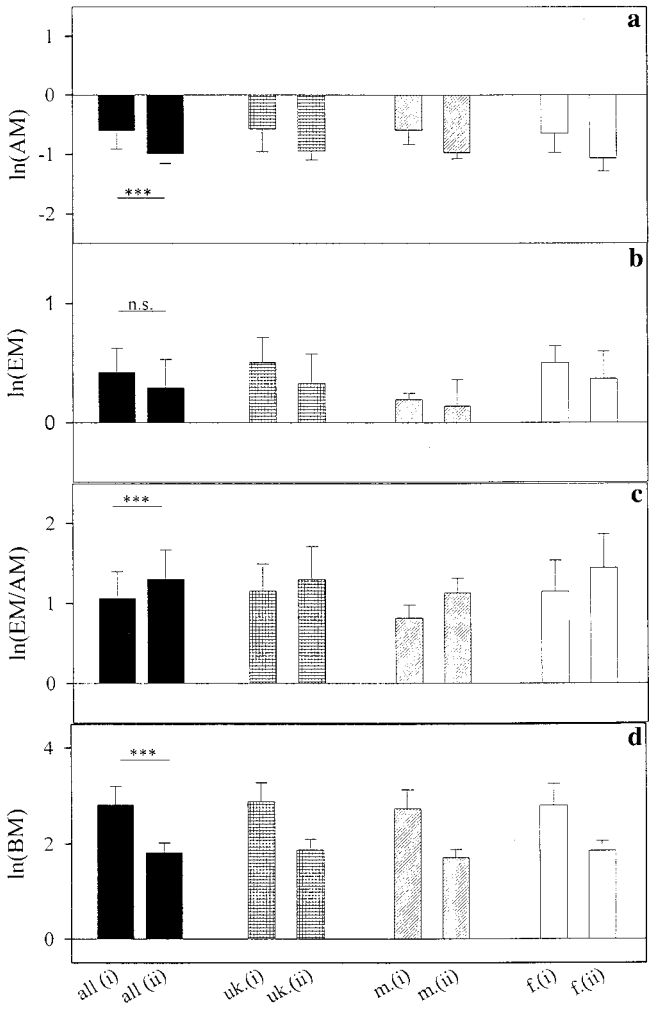

FIG. 3. Mean levels (+SD) of AM (a), EM (b), EM / AM (c), BM (d), during the two ontogenetic periods considered (i, days 1–20; ii, days 21–80) in all individuals ($n = 15$, "all," black bars), individuals of unknown sex ($n = 6$, "uk.," hatched bars), males ($n = 4$, "m.," hatched bars), females ($n = 5$, "f.," white bars). Original values (ng steroid / g feces) have been logarithmic (ln) transformed and arithmetic means are plotted here. Horizontal lines indicate significant differences between the two periods considered: ***$P < 0.000$; n.s., not significant.

DISCUSSION

Our data indicate a significant decrease in excreted levels of immunoreactive metabolites of 17β-OH-androgen, estrogen, and corticosterone during the first 10–20 days after hatching. Gonadal steroid metabolites were fluctuating more widely than corticosterone metabolites, which remained at low levels until fledging.

Results will be discussed with respect to ontogenetically relevant events. In particular, the following aspects will be considered: (1) how steroid hormones may be related to the timing of morphological and behavioral development from hatching to fledging and (2) the potential relevance of sex differences in posthatch hormone levels.

Patterns

High levels of steroids at birth are found in mammals (see Nelson, 1995) and in altricial and precocial birds at hatching (e.g., Ottinger and Brinkley, 1979; Ottinger and Bakst, 1981; Proeve, 1983; Tanabe *et al.*, 1983). In the present study, all steroid metabolites evaluated showed elevated levels at hatching and decreased thereafter (Fig. 1). Gonadal steroids revealed more fluctuations than BM, which remained at low levels until fledging after the first 20 days (Figs. 1 and 2). In other bird species (zebra finches, Adkins-Regan *et al.*, 1990; mallards, Tanabe *et al.*, 1983; domestic chickens, Tanabe *et al.*, 1986; starlings, Williams *et al.*, 1987) gonadal steroids show similar fluctuations during ontogeny, which may be related to structural processes during development (zebra finches, Proeve, 1983).

In the altricial zebra finch (Proeve, 1983), testosterone peaks at fledging and, together with estrogen,

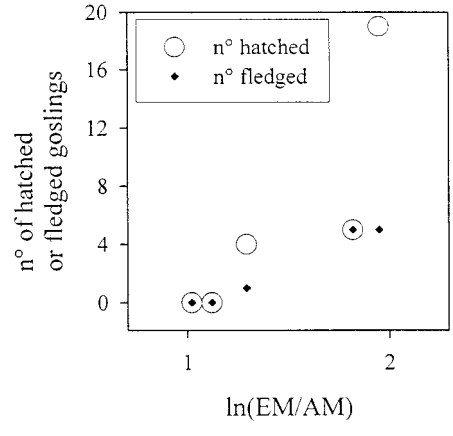

FIG. 4. Relationships between EM / AM and the number of hatched goslings (Kendall's $\tau = 0.949$, $n = 5$, $P = 0.023$) and between EM / AM and the number of fledged goslings (Kendall's $\tau = 0.894$, $n = 5$, $P = 0.037$) in five females.

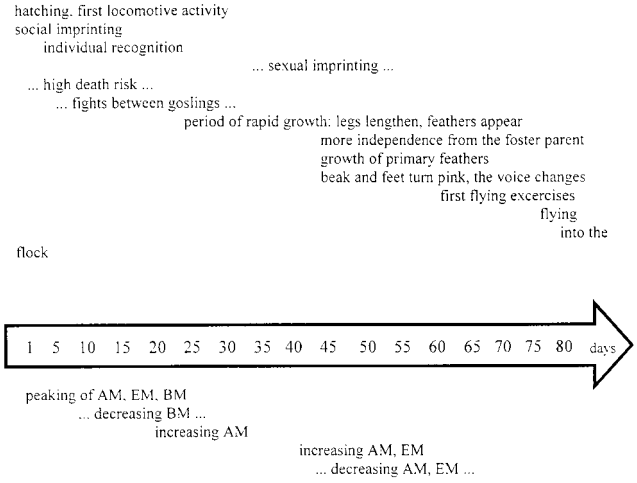

FIG. 5. Model (in accordance with Proeve, 1983) relating hormones to behavioral developmental processes in greylag geese.

seems to be related to the sensitive phase for sexual imprinting. In our geese, AM was increased at the age of 20 days (Fig. 1a), approximately when the main feathers start growing (Hudec and Rooth, 1995; Bisenberger *et al.*, 2000), and, together with EM (Fig. 1a), at the age of 40 days, when the goslings physically prepare for flight (i.e., running and flapping wings; Lorenz, 1988; Bisenberger *et al.*, 2000; Fig. 5). Although different developmental timing makes a direct comparison between altricial and precocial species difficult, the two species may be similar in the relationship between elevated androgen values and the acquisition of adult plumage and in the first attempts to fly. Based on experimental data (Bateson, 1979) and feather development, we furthermore suggest that the increase of AM and EM at age 40 days (Fig. 1a) could be related to the sensitive phase for sexual imprinting (Schutz, 1965; Lorenz, 1988; Fig. 5).

In contrast to sexual imprinting, social imprinting occurs within a few hours after hatching in greylag geese (Lorenz, 1937; Hess, 1973; Fig. 5) and involves learning and memory processes (Bateson, 1966). Cognitive functions are modulated also by adrenal steroids, such as corticosterone (McEwen and Sapolsky, 1995; e.g., glucocorticoids and memory formation in chicks: Sandi and Rose, 1994; Loscertales *et al.*, 1997). Therefore, high BM at hatching might affect the cognitive processes necessary for filial imprinting and other learning events. In particular, precocial birds have to adapt to the social and ecological complexities of a new environment immediately after hatching (Nakamura *et al.*, 1978; McNabb *et al.*, 1998; Pfeffer *et al.*, 2001). Within a few hours after hatching, greylag goslings have to imprint on their parents, feed on their own, and compete with their siblings for good brooding positions. This is also a critical period for survival, with only approximately 30% of the hatchlings fledging and the highest mortality occurring within the first 3 weeks of life (J. Hemetsberger, unpublished). Furthermore, a few days after hatching, fights between siblings occur and a rank order is established (Kalas, 1977; Lorenz, 1988). Therefore, coping with the immediate complexities of the social environment right after hatching might be related to high BM (Figs. 1b and 5).

Since glucocorticoids interact with thyroid hormones in regulating metabolism and corticosterone mobilizes energy, high levels of corticosterone may channel investment into behavior and, thus, inhibit growth (domestic chickens: Siegel *et al.*, 1989). Overlapping the pattern of BM with the growth curve obtained for greylag goslings (Fig. 1b), it appears that the goslings grow slowly as long as BM remains elevated (i.e., the first 15–20 days). In birds growth should be selectively pushed to a physiological upper limit since as long as young birds are unable to fly they are also at their most vulnerable to predation. Young birds grow about twice as fast as mammals of equal size (Björnhag, 1979), and precocial birds, which have more mature tissue at hatching, subsequently grow slower than altricial birds (Lesage and Gauthier, 1997). This would suggest that, at a speculative level, BM may promote cognitive processes and help to cope with a complex and new environment. However, as a trade-off, BM would delay growth during the days after hatching, even though a fast initial growth would significantly shorten the time until fledging.

Sex Differences

Androgen and estrogen are involved in sexual differentiation and later in the development of sexual behavior. Therefore, sex differences in the levels of these hormones might be expected during development. In adult geese females excrete higher levels of EM, whereas males produce higher AM throughout the entire annual cycle (Hirschenhauser *et al.*, 1999a). In our goslings, no significant sex difference in AM was found (Fig. 2a). This is consistent with studies in

chickens and starlings (Tanabe *et al.,* 1986; Williams *et al.,* 1987). In contrast, Tanabe *et al.* (1983) and Adkins-Regan *et al.* (1990) found that juvenile female mallards and zebra finches had higher levels of testosterone than males. Therefore, our results could be characteristic for the species, but may also be due to our small number of individuals of known sex (four males, five females). With regard to sex differences in EM (Fig. 2b), our results are consistent with those from Bercovitz *et al.* (1985) and Tanabe *et al.* (1986) in the precocial domestic chicken, in which female hatchlings show higher levels of estrogen than males. A similar study on the altricial zebra finch did not find such a difference (Adkins-Regan *et al.,* 1990). In birds, the heterogametic female sex requires exposure to estradiol to differentiate into a physiological female (Ottinger, 1989; McNabb *et al.,* 1998). From hatching to fledging, female greylags excreted more EM than males. This suggests a major role of EM in the differentiation and development of female phenotype. Concerning BM, no significant difference could be found between males and females, which is consistent with the findings in ducks (Tanabe *et al.,* 1983), chickens (Tanabe *et al.,* 1986), and adult greylag geese (Hirschenhauser, 1998).

Recently, several studies have centered on early steroid hormones and their effects on later life history stages, particularly in modulating sociosexual behavior. Experiments in zebra finches showed that posthatch treatment with estradiol benzoate may affect female mate choice (Adkins-Regan and Ascenzi, 1987; Mansukhani *et al.,* 1996). In adult greylag geese, the circannual testosterone covariation within pairs is positively correlated with reproductive success and has, therefore, been considered a correlate of pair bond quality (Hirschenhauser *et al.,* 1999b). Our results suggest that early ratios of EM / AM may be related to later reproductive success in female geese (Fig. 4) and, therefore, to female quality.

In conclusion, our data suggest a major role for steroid hormones in the fine tuning and modulation of development in a precocial bird (Fig. 5). BM may be especially involved in an energy allocation trade-off (behavior versus growth) during the first days of life. Early gonadal steroids appear to be related to sexually related events, such as sexual imprinting and later even to female reproductive success.

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